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FOREWORD

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In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

CSH In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


 2/20/97
PI - Signature Date

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A. INTRODUCTION

The following is a summary of activities performed under the auspices of this grant, with the Yale University personnel (paid and unpaid) involved in this work listed next to each heading.

The requested funds, previously supplemented by NIH and WHO monies, supported research of the World Reference Center for Arboviruses which: (a) identifies and characterizes approximately 80 suspected arboviruses per year, submitted from U.S. and overseas laboratories, (b) develops new techniques for rapid diagnosis and for characterization of arboviruses and arenaviruses, (c) prepares and distributes reference immune reagents and specific PCR protocols and primers, (d) prepares virus stocks for distribution through WHO regional reference centers and the American Type Culture Collection, (e) prepares and distributes virus antigens, (f) carries out limited virus serosurveys, and (g) disseminates information through WHO, the American Committee on Arboviruses, and by reports in scientific journals.

Emphasis was placed on specific subprojects, which include: (a) nucleotide sequence analysis of flavivirus and arenavirus RNA for molecular epidemiological study of strains, (b) development of viral inactivating agents, for treatment of clinical samples, to render them non-hazardous, (c) use of the extensive reagent bank, to identify emerging viruses by antigenic and genetic methods, and (d) updating maintenance and cataloguing methods for the virus collection.

B. ACTIVITIES

1. World Reference Center: Shirley Tirrell-Peck and Joann Zamparo

a. Reagents shipped

The following is a list of recipients of viruses, antigens, and/or antibodies requested from the Reference Center collection:

<u>Date</u>	<u>Recipient</u>	<u>Institution</u>
1/16/96	S. Oberste	USAMRIID, Frederick, MD
1/23/96	R. Shope	Univ. Texas, Galveston, TX
2/13/96	S. Oberste	USAMRIID, Frederick, MD
2/18/96	R. Shope	Univ. Texas, Galveston, TX
3/12/96	L. Walters	Univ. Alaska, Fairbanks, AK
3/12/96	P.G. Jupp	Natl. Inst. Virology, South Africa
3/19/96	S. Weaver	Univ. Texas, Galveston, TX
3/26/96	C. Ramos	Natl. Inst. Public Health, Mexico
4/8/96	R. Shope	Univ. Texas, Galveston, TX
4/15/96	B. Hjelle	Univ. New Mexico, NM

4/18/96	C. Ramos	Natl. Inst. Public Health, Mexico
4/22/96	J. Morvan	Pasteur Inst., Paris, France
5/6/96	R. Shope	Univ. Texas, Galveston, TX
5/13/96	J. Smith	USAMRIID, Frederick, MD
5/22/96	L. Walters	Univ. Alaska, Fairbanks, AK
5/30/96	J. Roehrig	CDC, Fort Collins, CO
6/18/96	R. Shope	Univ. Texas, Galveston, TX
6/24/96	J. Morvan	Pasteur Inst., Paris, France
6/27/96	A. deSilva	Medical Res. Inst., Colombo, Sri Lanka
7/15/96	S. Zaki	CDC, Atlanta, GA
7/16/96	R. Shope	Univ. Texas, Galveston, TX
7/17/96	L. Walters	Univ. Alaska, Fairbanks, AK
7/31/96	B. Innis	WRAIR, Washington, DC
8/5/96	R. Shope	Univ. Texas, Galveston, TX
8/20/96	A. Rothman	Univ. Massachusetts, MA
8/21/96	S. Zaki	CDC, Atlanta, GA
8/26/96	S. Weaver	Univ. Texas, Galveston, TX
8/28/96	N. K.-Thasan	WRAIR, Washington, DC
9/10/96	M. L.-Vazquez	Univ. Guerrero, Mexico
9/11/96	N. Monteny	ORSTOM, Sinaloa, Mexico
9/18/96	R. Novak	Illinois Nat. Hist. Survey, IL
10/14/96	L. Walters	Univ. Alaska, Fairbanks, AK
10/21/96	B. Wolf	NJ State Dept. Public Health
10/22/96	F. Lopez	Foothill s Res. Center, CA
11/4/96	P. Mason	USDA, Plum Island, NY

Virus shipment costs rose disproportionately because of new AITA requirements for special shipping containers (Safe-T-Pak), only one carrier licensed to transport etiologic agents (Federal Express), additional fees and paperwork, and prior certification or acknowledgement of the availability of laboratory facilities meeting biohazard containment levels (e.g., CDC and/or USDA permits).

b. DNA primers

Synthetic DNA primers were prepared, according to publications of ours and others, for PCR protocols to detect, serotype, and genotype dengue viruses from patient serum samples. These primers were shipped to 6 laboratories (in Mexico, Venezuela, India, and Sri Lanka) for routine diagnostic procedures in national and regional reference laboratories.

c. Virus identification

This activity comprised most of the work done under the auspices of this grant. This work became especially important in arbovirus surveillance in the northeastern part of the United States, during the period June-Nov., 1996. Because of our unique expertise and location, the Yale Arbovirus Research Unit was responsible for the detection and identification of encephalitis viruses in mosquitoes collected in Rhode Island and Connecticut. Eastern equine encephalitis (EEE) virus was isolated from 93 of 1800 mosquito samples collected at various sites within these two

states (virus was also detected in Massachusetts and New York). Both State Health Departments relied on our test results and rapid turnaround for the formulation of control policies to protect the public's health. Control methods included mosquito abatement with insecticides of various types, closing of recreational parks, and public awareness campaigns, to reduce exposure. Fortunately, probably because of this rapid response, no human cases of encephalitis occurred during this period. There were deaths in pheasants and emus that were confirmed to be due to EEE during this period, but none in horses.

During the course of this reporting period, some (5) human clinical samples were received from Yale-New Haven and Morristown Hospitals, for arbovirus testing. Most of these cases were diagnosed as probable viral encephalitides, but all were negative in a standard battery of tests. Others were received from suspected dengue cases; these were negative also. Other tests involved detecting specific antibodies (by ELISA and PRNT) to several arboviruses in samples from Alaska, as part of an ongoing, long-term project to detect arbovirus transmission in Alaska natives.

d. Collection maintenance

This activity involves virus and antibody maintenance in freezers, having collection information available in electronic form, and responding to reagent requests via electronic mail. In addition to maintaining one of the largest collections of arboviruses in the world, YARU also adds to the collection as viruses emerge; this allows us to have a full spectrum of diagnostic reagents available, for the immediate identification of new viruses. During this report period, a total of 9 low temperature freezers were purchased, to replace nonfunctional units and to avoid failures of freezers which had been in use for over 20 years. Consolidation of samples in larger freezers also allowed us to meet current biosafety guidelines, so that we could store all viruses under lock, in one room. The YARU virus collection files were transferred to a new computer (PC 486SX), with new software (MS Excel & dBASE), so they are more accessible to users; we can now transfer all virus information in Zip drive disks. The Reference Center laboratory also has its own electronic mail connection, to receive requests and to notify Yale Biosafety offices and others of outgoing shipments of viruses.

e. Personnel training

Both Research Associates involved in the Reference Center activities attended a special course on how to meet new virus shipping requirements (AITA, CDC/NIH, FedEx, USDA, Yale Biosafety) so that we could respond promptly to requests from others. In addition, eight Yale University students and other investigators (from Mexico, Venezuela, etc.) were trained in basic protocols used for virus identification (e.g., PCR, ELISA, HI).

2. Field Surveillance for Arboviruses

a. Turkey - Mark L. Wilson

As part of related studies on arthropod-borne disease agents in an area of southeastern Turkey undergoing extensive environmental change from a major irrigation project there, we collected mosquitoes in hopes of isolating arboviruses. This area of the Near East, which includes northern Syria, western Iraq, and Iran has not been surveyed recently due to political and logistic problems. We anticipated that the region of southeastern Turkey, bordering these three countries, presented an opportunity to attempt arbovirus isolations from the natural vector fauna of the region.

During June - August 1996, Pia MacDonald (Yale MPH student) worked with Turkish medical entomologists (Dr. H. and M. Kasap) in an attempt to capture and preserve mosquitoes for isolation efforts. CDC light traps were set in various sites during this the hot/dry season to capture adult insects. Unfortunately, only a few mosquitoes were captured (mostly *Anopheles* species), even though traps were set in habitats likely to be suitable for adults (near streams, inside dwellings, near reservoirs, etc.). In addition, it was not feasible to preserve these few specimens captured in liquid nitrogen. Nevertheless, the identity of these few *Anopheles* species was determined, and data on the incidence of malaria by provinces in the region was calculated. This work was part of Ms. MacDonald's MPH thesis.

Additional studies of sandfly vectors of cutaneous leishmaniasis were undertaken, in part through the use of CDC traps purchased from the grant. These observations were designed to evaluate how habitat characteristics derived from satellite image data, and related to demographic and environmental variables, might influence transmission. Results of these studies on malaria and cutaneous leishmaniasis were presented at the 1996 annual meeting of the American Society of Tropical Medicine and Hygiene, and have been submitted for publication or are in preparation.

b. Sri Lanka - Aravinda de Silva

This project involved collecting serum from toque macaque (*Macaca sinica*) monkeys in Polonnaruwa, Sri Lanka, to attempt to identify a dengue-like flaviviruses that had presumably infected them (Peiris et al., *J. Med. Primatol.* 22:240-245, 1993). Previous studies had shown that, of 68 animals sampled in 1987, 35% had dengue type 2 antibody (PRNT) titers of over 1:160. No virus isolations had been made and the infecting virus could not be identified.

We relied on newer serologic (IgG ELISA) and genetic (PCR) techniques to try to determine the identity of the infecting flavivirus. During July-Oct. 1995, 246 serum samples were collected by our collaborator, Dr. Wolfgang Dittus, of the National Zoological Park, Smithsonian Institution, Washington, DC,

from toque macaques ranging in age from 4 mos. to 33 yrs., from the same population group as that reported earlier. The ELISA test was done using various antigens from flaviviruses which had been reported to circulate in Sri Lanka and India (dengue, Japanese encephalitis, yellow fever, West Nile, Zika); only dengue-reactive antibodies were detected. The results showed that only animals over the age of 9 years had antibodies to a dengue-like virus and the study of several animals in the 8-9 year range allowed us to pinpoint the time of infection during Aug. 1986-Jan. 1987. Because none of the younger macaques had antibodies, this virus seemed to have disappeared from this ecological niche during the period 1987-1995. PCR tests on several of the positive sera were negative and we realized that the samples had previously been heat-inactivated, probably destroying any available viral RNA template. We are presently trying to obtain serum samples from 1986 or earlier, which have been stored at -20 C or below, without heat inactivation, so that we may attempt to enzymatically amplify the infecting virus and definitely determine its identity. One trip was made to the National Zoo, in Washington, to go through the serum collection held there, to find the appropriate samples for PCR, but this was unsuccessful. We are still checking for aliquots in collections stored at Columbia University and at a private company in Texas.

The results of this study could potentially provide very important information concerning the origin of epidemic strains of dengue virus and whether these viruses are exchanged between human and non-human primate populations in an island such as Sri Lanka. There have been numerous human epidemics there since the 1970's, although most have been limited to the western, urban areas of the country (Colombo). Because the macaque population under surveillance is limited to the north-central area of the country, we hypothesized that this possible dengue virus could represent another example of a sylvatic strain of dengue which does not seem to infect humans.

c. Venezuela - Katrin Leitmeyer

During the period June 9-18, 1996, a trip was made to Venezuela, to collect samples of Guanarito virus from humans and rodents. This project involved obtaining epidemiologic and clinical information on human hemorrhagic fever cases and ecologic information on infected rodents (*Zygodontomys brevicauda*), to determine the geographic and temporal distribution of this virus in Venezuela. This work was done in collaboration with the National Institute of Hygiene, in Caracas. Another objective was to obtain unpassaged or low passage virus samples, for optimization of the PCR test being developed for specific identification of arenaviruses (see below).

The samples obtained during this visit included 9 human serum aliquots from hemorrhagic fever cases hospitalized during the period Feb. 1991 - Mar. 1996, in Portuguesa State. Rodent samples included 26 virus isolates from *Z. brevicauda*, collected

in the same area, during the period Feb. 1994 - Feb. 1995. We are currently determining the nucleotide sequence of amplified viral RNA, to understand the population dynamics of this virus in the rodent reservoir(s) and its human host. This information will eventually provide a better estimate of Guanarito virus mutation frequencies in nature, an important consideration in the design of vaccines.

3. Development of Rapid Diagnostic Reagents

a. Hemorrhagic Fever PCR - Katrin Leitmeyer

The goal of this project was to develop a broadly reactive and non-hazardous PCR protocol for detection of hemorrhagic fever viruses (arenaviruses). Patient and rodent serum was first treated with a monophasic solution of phenol and guanidine isothiocyanate; this step inactivates any infectious agents while conserving viral nucleic acids for subsequent enzymatic amplification (shift from BL4 to BL2 containment levels). The viral RNA was extracted and reverse transcribed using a commercial kit and a specially-designed DNA primer (ARE/3'end: CGCACAGTGGATCCTAGGC, 19-mer). The resulting cDNA template was used in a PCR, with the addition of a virus-sense primer (ARE/2595V: CTRARRTTRTANCCWGADATRT, 22-mer), to generate a 800 bp fragment, after 40 thermal cycles. Different PCR conditions were tested (primers, pH, temperature, salt concentration, etc.) to determine optimal yields. These products were sequenced manually with commercially available kits.

The resulting nucleotide sequences were used to distinguish two arenaviruses (Guanarito and Pirital) which are transmitted simultaneously in *Sigmodon alstoni* rodents in Venezuela. This rapid diagnostic test was used to investigate the temporal and geographic range, host species, and human pathogenicity of these and other arenaviruses in this country. This test was also used to distinguish the etiology of human cases of hemorrhagic fever, specifically to exclude other viral agents such as dengue, yellow fever, and hantaviruses.

b. Virus inactivation in serum - Shirley Tirrell-Peck

In collaboration with Dr. Thomas Monath, of Pentose Pharmaceuticals Inc., we began testing the virus inactivation potential of several polymers of ethylenimine (i.e., pentose). We chose Venezuelan equine encephalitis (VEE) viruses as models for testing inactivation and structural protein (antigenic) stability because of our ample experience and the availability of numerous monoclonal antibodies with very sensitive reactivities with these viruses. Several strains of VEE viruses were successfully inactivated by a pentose trimer, when placed in normal human serum, while retaining all detectable antigenic sites. These studies are continuing, so that we may optimize treatment conditions to suit application of these inactivating agents in a clinical setting. The potential use of these agents

includes: treatment of blood or other patient fluid samples, to render them non-hazardous for further examination of clinical parameters and virus detection; use of these agents for vaccine production, especially for emerging viruses, for rapid turnaround of killed virus immunogens; elimination of adventitious viruses in many commercial or industrial preparations.

4. Molecular Epidemiology

a. Arenavirus phylogeny - Jean-Paul Gonzalez

Most of the activity for this project was limited to the study of Sabiá virus, due to limitations on the growth of these agents in our facilities, because of biological safety concerns. We had already prepared numerous aliquots of extracted viral RNA of several arenaviruses, but our attempts to amplify some of these by PCR were unsuccessful.

Sabiá virus, one of five arenaviruses from South America known to cause hemorrhagic fever in humans, emerged in 1990 when it was isolated from a fatal case in Sao Paulo, Brazil. Subsequently, it has caused two laboratory-acquired infections. Its natural distribution and host are still unknown. Using viral RNA and multiple polymerase chain reaction products as templates, the nucleotide sequence of the small (S) RNA segment of Sabiá virus, which codes for the nucleocapsid and glycoprotein precursor, was determined. This virus shares an ambisense genome in common with other arenaviruses, although it has a unique predicted three stem-loop structure in the S RNA intergenic region. Phylogenetic analysis of a portion of the N gene sequence confirmed that this virus is distinct from all other members of the arenavirus family and that it shares a progenitor with Junin, Machupo, Tacaribe, and Guanarito viruses. We were also able to establish that the analysis of only 730 nucleotides was required for obtaining reliable phylogenies and this approach could potentially be used to identify and classify subsequent emerging arenaviruses. This approach was adapted to a rapid PCR protocol which could distinguish arenaviruses from several countries in the Americas.

b. Dengue virus pathogenicity - Rebeca Rico-Hesse

Ongoing studies on the molecular evolution of dengue type 2 viruses provided new information on the origin of more pathogenic viruses belonging to this serotype. Other research projects done in Thailand (sponsored by NIH) have also contributed to a better understanding of severe dengue pathogenesis. Our working hypothesis is that both host (immune status) and viral (genetic) factors determine the potential for dengue fever progressing to hemorrhagic fever in the infected individual.

The recent emergence and spread of dengue hemorrhagic fever in the Americas has been a major source of concern. Efforts to control this disease are dependent on understanding the

pathogenicity of dengue viruses and their transmission dynamics. Pathogenicity studies have been hampered by the lack of in vitro or in vivo models of severe dengue disease. Alternatively, molecular epidemiologic studies which associate certain dengue virus genetic types with severe dengue outbreaks may point to strains with increased pathogenicity. The comparison of nucleotide sequences (240 bp) from the E/NS1 gene region of the dengue virus genome has been shown to reflect evolutionary relationships and geographic origins of dengue virus strains (*Virology* 174:479-493, 1990). This approach was used to demonstrate an association between the introduction of two distinct genotypes of dengue type 2 virus and the appearance of dengue hemorrhagic fever in the Americas. Phylogenetic analyses suggest that these genotypes originated in Southeast Asia and they displaced the native, American genotype in at least 4 countries. Vaccination and other control efforts should therefore be directed at decreasing the transmission of these "virulent" genotypes.

5. Therapeutic Interventions for Exotic Viral Infections

There was no activity in this area; fortunately, YARU clinical personnel did not have to respond to emergencies of this type. Drs. Michelle Barry and Frank Bia therefore did not collect salaries for this activity.

C. CONCLUSIONS

In addition to maintaining one of the largest collection of reference viruses in the world, the Reference Center served functions critical for maintaining the public's health and provided important information concerning the origin, transmission and control of arboviruses. We provided training and reagents to both national and foreign laboratories, so that they might also contribute to the detection and control of these viruses. The results reported here and those obtained by others are important in understanding the epidemiology of viruses exotic to the United States, including those considered to be emerging in other areas of the world and therefore potentially hazardous to military and civilian personnel of this country.

D. BIBLIOGRAPHY

1. Publications

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Wang, E., Weaver, S.C., Shope, R.E., Tesh, R.B., Watts, D.M., and A.D.T. Barrett (1996). Genetic variation in yellow fever virus: duplication in the 3' noncoding region of strains from Africa. *Virology* 225:274-281.

Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J.R., de Mesa, M.T., Nogueira,

R.M.R., and Travassos da Rosa, A. (1997). Origins of dengue serotype 2 viruses associated with increased pathogenicity in the Americas. *Virology*. In press.

Alptekin D, Kasap M, Kasap H, Aksoy S, Wilson ML. Sandflies (Diptera: Psychodidae) associated with an epidemic of cutaneous leishmaniasis in Sanliurfa, Turkey. *J. Med. Entomol.* (submitted).

2. Meeting Abstracts

Rebeca Rico-Hesse

Am. Soc. Trop. Med. Hyg., Dec. 1996: "Evolution of dengue type 2 viruses associated with increased pathogenicity in the Americas".

International Symposium on Factors in the Emergence of Arbovirus Diseases, Annecy, France, Dec. 1996: "Molecular evolution of dengue type 2 viruses".

Mark L. Wilson

Am. Soc. Trop. Med. Hyg., 1996: "*Phlebotomus* sandflies associated with epidemic cutaneous Leishmaniasis in Sanliurfa, Turkey"; "Epidemic reemergence of cutaneous Leishmaniasis in Saliurfa, Turkey: Epidemiological characterization and hypotheses".

E. LIST OF PERSONNEL (receiving salary)

Mark L. Wilson, Associate Professor
Katrín Leitmeyer, Postdoctoral Associate
Shirley Tirrell-Peck, Senior Research Associate
Joann Zamparo, Research Associate
Sherry Calcasaco, Secretarial Assistant
Mary Ganues, Laboratory Aide
E. Kruglov (filling in for M. Ganues)